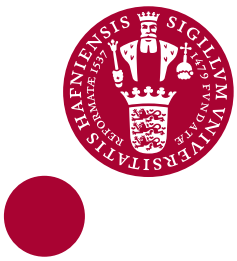

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Published in:
ACS Omega

DOI:
10.1021/acsomega.9b00098

Publication date:
2019

Document version
Publisher's PDF, also known as Version of record

ACS OMEGA

Article

Cite This: ACS Omega 2019, 4, 5968−5982

http://pubs.acs.org/journal/acsodf

Structure−Function Characteristics and Signaling Properties of Lipidated Peptidomimetic FPR2 Agonists: Peptoid Stereochemistry and Residues in the Vicinity of the Headgroup Affect Function

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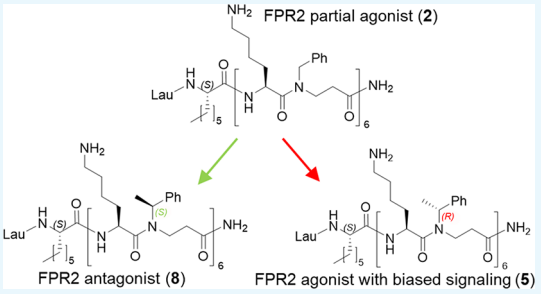
Supporting Information

ABSTRACT: Formyl peptide receptor 2 (FPR2) plays important roles in inflammation. In the present study, 20 analogues of the FPR2-selective lipidated α-peptide/β-peptoid agonist Lau-[(*S*)-Aoc]-[Lys-βNPhe]$_6$-NH_2 were generated, which allowed two novel subclasses of more potent FPR2 agonists to be distinguished. Critical factors influencing FPR2 recognition comprise the presence of β-peptoid phenylalanine-like residues (i.e., βNPhe, βNspe, or βNrpe) in the peptidomimetic tail, configuration of the 2-aminooctanoic acid (Aoc) in the headgroup, and the length of the N-terminal fatty acid. Intriguingly, a single βNrpe residue in the vicinity of the N-terminus (i.e., Lau-[(*S*)-Aoc]-Lys-βNrpe-[Lys-βNPhe]$_5$-NH_2) proved to increase the agonist potency, whereas the βNspe-containing analogue was a weak FPR2-selective antagonist. Another subclass displaying potent agonism comprised analogues possessing two α-amino acids vicinal to the headgroup. The optimized FPR2-activating lipidated peptidomimetics exhibited biased signaling: PLC-PIP_2-Ca^{2+} signaling was activated, but without recruitment of β-arrestin or induction of chemotaxis. These FPR2-interacting compounds are considered to be useful tools in future studies of receptor−ligand interactions.



INTRODUCTION

Recruitment of neutrophils to inflammatory sites is directed by host- or pathogen-derived danger molecules recognized by G-protein-coupled receptors (GPCRs), for example, receptors for platelet-activating factor (i.e., PAFR), adenosine triphosphate (i.e., $P2Y_2R$), and formyl peptide receptors FPR1 and FPR2. The third FPR subclass (i.e., FPR3) is not expressed by human neutrophils, and its biological functions are not yet known.[1] FPRs are high-affinity receptors for formyl peptides, being danger molecules of bacterial and mitochondrial origin, albeit these receptors also recognize a large number of non-formylated peptides and small-molecule ligands, representing different chemical classes.[2−4] FPRs are important not only for neutrophil recruitment and initiation of inflammation but also for resolution of such processes. Thus, aberrant FPR expression is associated with a wide range of immunity-mediated diseases, for example, atherosclerosis, cancer, neurodegeneration, and sepsis,[5,6] and thus, FPRs constitute attractive therapeutic targets. Consequently, an increasing number of FPR ligands with therapeutic potential have been identified and characterized in recent years.[3,4]

A major complication in therapeutic use of peptide-based ligands is their intrinsic susceptibility to in vivo degradation by peptidases, conferring low bioavailability. Hence, peptidomimetics (partly composed of non-natural residues) that resist enzymatic degradation constitute favorable alternatives. Stable α-peptide/β-peptoid hybrid oligomers displaying an alternating design with cationic α-amino acids and aromatic hydrophobic β-peptoid residues (Figure 1) have been found to mimic the biological activities of peptides, such as microbial killing, reduction of biofilm formation, and immunomodulation. This class of peptidomimetics has proved to constitute a promising source of lead compounds for the development of potential pharmaceuticals within these therapeutic areas.[7−9]

FPRs belong to the superfamily of GPCRs that mediate fundamental cellular responses upon agonist binding, with subsequent G-protein-dependent signal transduction.[6] Both pro- and anti-inflammatory responses are mediated by FPRs,[5]

Received: January 11, 2019
Accepted: March 13, 2019
Published: March 28, 2019

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

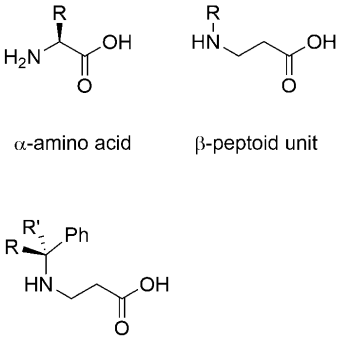


Figure 1. Nomenclature of structural units in the peptidomimetics investigated.

and this dual functionality appears to be in line with the "biased signaling" concept, often termed "functional selectivity".[10,11] Classically, activation of a GPCR signaling pathway initiates the dissociation of a heterotrimeric G-protein, triggering production of multiple downstream secondary messengers, for example, phospholipases and protein kinases. The resulting signaling cascade is terminated by recruitment of β-arrestin to the GPCR, a coupling that sterically blocks further G-protein recruitment/signaling.[12] Recent research has highlighted that β-arrestins, besides this involvement in termination, also possess a G-protein-independent signaling capacity.[13,14] The concept of biased signaling states that a certain ligand may selectively activate one (or a few) of several signaling pathways possible for a given receptor: that is, an activated receptor may induce signals both downstream from the G-protein and downstream from β-arrestin, or signaling may only occur through one of these pathways.[10,15] In line with this, we have recently shown that an FPR2-selective agonist, $F2Pal_{10}$, belonging to the pepducin-type lipopeptides, in fact is a biased FPR2 agonist that activates PLC-PIP_2-Ca^{2+} signaling and assembly of the superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the absence of β-arrestin recruitment.[16] We have earlier identified both neutrophil-inhibiting and -activating lipidated peptidomimetics (e.g., **1** and **2**, respectively; Figure 2), for which it was shown that FPR2 is the preferred receptor. The presence of a hydrophobic N-terminal headgroup as well as the β-peptoid units in the tail moiety were essential for the biological activities of these lipidated peptidomimetics.[17−19] Nevertheless, several structural features of these ligands remain to be investigated in order to delineate the more precise requirements for the optimal interaction with FPRs, in particular, the factors determining whether an agonistic or inhibitory receptor-selective outcome results from the interaction.

A major purpose of the present study was identification of structural determinants in lipidated α-peptide/β-peptoid hybrid peptidomimetics that are of importance for the FPR2-dependent activation of neutrophils by agonists resembling compound **2**. This included comparison of the FPR2 interaction of **2** with that of 3 known and 20 novel analogues (i.e., **3**−**25**). The present structure−function optimization of compound **2**, a previously described cross-species FPR2 agonist, resulted in identification of two subclasses of more potent FPR2 agonists. The full agonistic activity was found to rely not only on the presence of a fatty acid of appropriate length and the stereochemistry of the adjacent 2-amino-octanoic acid (Aoc) residue, but also on the nature of the two vicinal residues as well as the presence/absence of α-chirality in the peptoid residues of the tail region. Moreover, our data disclose a propensity for biased signaling of FPR2 agonists belonging to this class of lipidated peptidomimetics that trigger the PLC-PIP_2-Ca^{2+} signaling pathway without recruitment of β-arrestin or induction of chemotaxis.

RESULTS AND DISCUSSION

Introduction of α-Chiral β-Peptoid Units Influences the Type of Activity and Potency. Through screening of a focused library of lipidated α-peptide/β-peptoid hybrids, Lau-[(*S*)-Aoc]-[Lys-βNPhe]$_6$-NH_2 (**2**; Figure 2) was previously identified as the first FPR2-selective peptidomimetic agonist, and the FPR2 preference was confirmed by using FPR2 overexpressing cells.[17] Compound **2** is a partial FPR2 agonist that induces release of superoxide with a slightly lower potency and efficacy in comparison to that of the prototypical peptide FPR2 agonist WKYMVM.[17] Incorporation of shorter or longer N-terminal fatty acids (to give **3** or **4**, respectively; Table 2) resulted in reduced or unaffected potency as compared to that of **2**, while FPR2 activation proved to be dependent on an (*S*)-configurated N-terminal Aoc residue.[17] These findings prompted us to investigate the influence of chirality in other parts of the molecule, and therefore, several analogues were designed as depicted in Figure 2.

Introduction of chirality in the β-peptoid unit by incorporation of βNrpe (to give **5**) conferred increased efficacy in triggering superoxide release because **5** induced a higher maximum response, but similar efficacy when compared to the response induced by WKYMVM, and is therefore considered to be a full agonist for the receptor (Figure 3A, and Supporting Information, Figure S1). Furthermore, analysis of the dose−response curves showed that compound **5** is ∼3-fold more potent than **2**, with EC_{50} values of 58 nM (95% CI: 51−66) and 167 nM (95% CI: 142−197), respectively (Figure 3B). The response induced by compound **5** was mediated through FPR2 as inferred by the inhibition profiles, obtained when applying well-characterized selective FPR1 and FPR2 antagonists (see the list of prototypical ligands in Table 1). Thus, the activity of **5** was completely abolished by preincubation with two FPR2-selective antagonists, i.e., PBP_{10} and the earlier identified peptidomimetic inhibitor **1** (Figure 4A,B). In contrast, the FPR1-selective antagonist cyclosporine H (CysH) did not influence the response induced by **5**. Furthermore, the FPR2 selectivity of **5** was confirmed by receptor desensitization experiments, showing that neutrophils activated by the FPR2 agonist WKYMVM were desensitized to a subsequent dose of **5** and of WKYMVM. In contrast, neutrophils stimulated by fMLF were fully responsive to a subsequent dose of **5** but not to fMLF (Figure 4C,D).

The influence of peptoid stereochemistry on activity was further investigated by examining analogue **8** in which the α-chiral βNrpe peptoid residues were replaced by βNspe units,

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

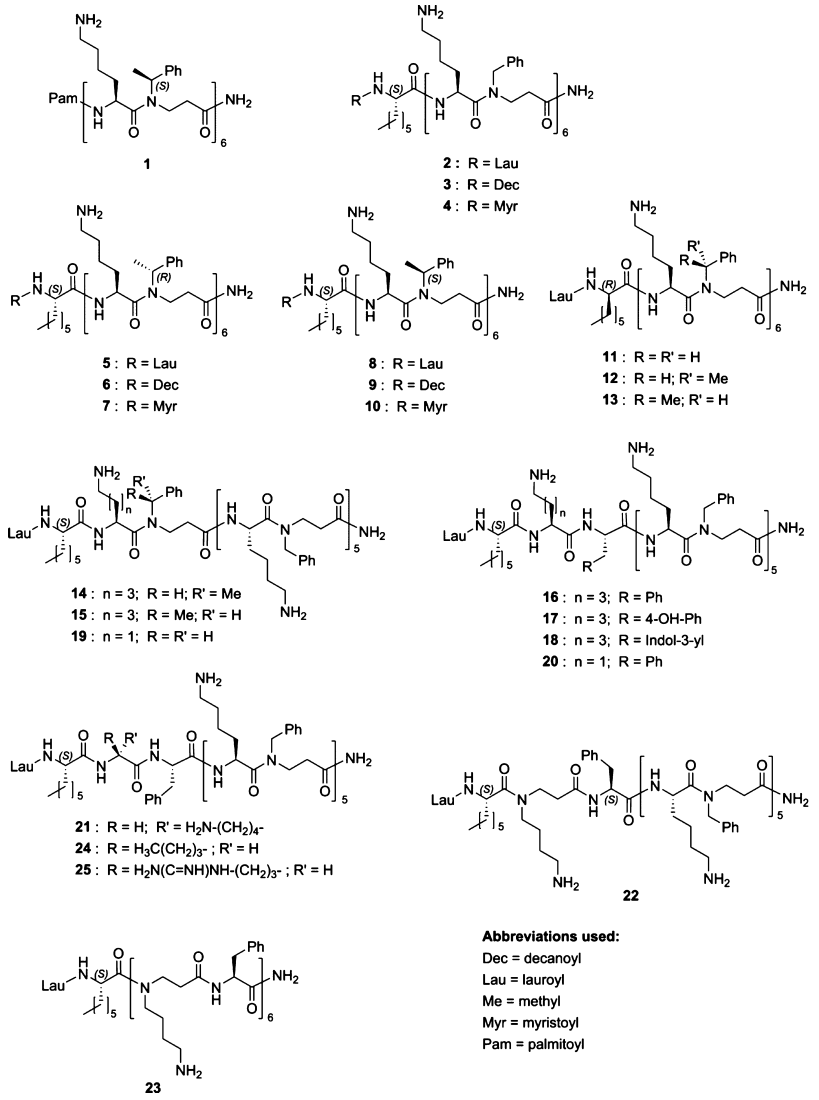


Figure 2. Chemical structures of peptidomimetics investigated as potential FPR2 agonists.

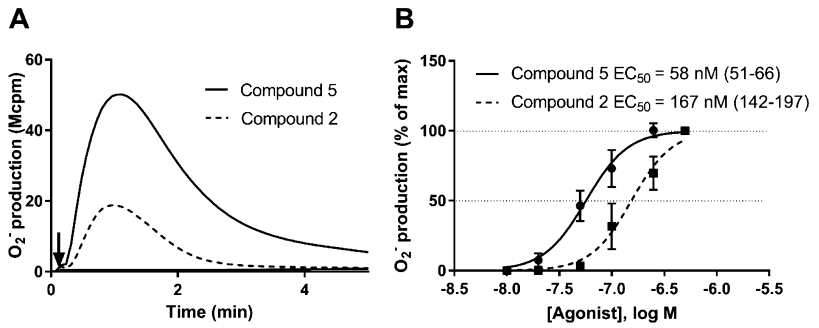


Figure 3. Compound **5** activates human neutrophil NADPH oxidase as a full agonist. The production of superoxide anions was measured by an isoluminol-enhanced chemiluminescence system (see Experimental Section). Neutrophils (10^5 cells/mL) were preincubated (5 min at 37 °C) and then stimulated by compound **2** or **5**, after which the release of superoxide anions was recorded continuously. (A) Representative response curves out of at least three independent experiments for compounds **2** and **5** (at 500 and 250 nM, respectively, that give maximal response). Abscissa: time of study (min); ordinate: superoxide production (10^6 × counts/min; Mcpm). (B) Dose-dependent superoxide release induced by compounds **2** and **5**. Data are presented as normalized peak response values (mean ± SD; n = 3 for compound **5**, and n = 5 for compound **2**), which differ between the two compounds (see subfigure A). The graph furthermore shows the fitted curve as well as the calculated EC_{50} (95% CI).

displaying the opposite side-chain stereochemistry (see Figure 1). Interestingly, **8** lacked the ability to confer activation of neutrophil NADPH oxidase (Figure 5A). Generally, the level of superoxide released by neutrophils depends on the cellular state examined, being either naïve (i.e., low-responding) or primed (i.e., high-responding). The activity of **8** was examined on cells in the primed state, achieved by incubating naïve cells with tumor necrosis factor-α (TNF-α) for 20 min at 37 °C. No

DOI: 10.1021/acsomega.9b00098
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Table 1. Established FPR Ligands Used in the Present Study and Their Activity Profile

name	activity and receptor selectivity	references
WKYMVM	FPR2 agonist	20
fMLF	FPR1 agonist	21,22
PBP_{10}[a]	FPR2 antagonist	23
CysH[b]	FPR1 antagonist	24

[a]RhB-QRLFQVKGRR-OH, where RhB = rhodamine B. [b]CysH = cyclosporine H.

superoxide release was inducible by **8** from such primed cells (Figure 5A), thus further confirming the lack of agonistic effect of **8**.

Our experience gained from the work on FPR2-interacting lipopeptides belonging to the pepducin family suggests that subtle structural modification, for example, a single exchange of an amino acid, may result in a switch of FPR2 ligands from agonist to antagonist.[25,26] On this basis, the inhibitory effect of **8** on FPR2 was examined, showing that **8** dose-dependently, but incompletely, inhibited the response generated by the FPR2-selective agonist WKYMVM with an IC_{50} value of 0.29 μM (CI: 0.22−0.37 μM; Figure 5B and inset herein). The ability of peptidomimetic **8** to produce a transient increase in the concentration of cytosolic Ca^{2+}, a very early downstream signaling event following FPR activation, was also investigated. Expectedly, **8** proved unable to trigger a transient Ca^{2+} response by itself. The receptor preference of **8** for FPR2 over FPR1, measured as its inhibitory effect in the NADPH oxidase assay, was confirmed because **8** also blocked the WKYMVM-induced Ca^{2+} transient (Figure 5C). By contrast, the transient increase in the intracellular calcium level, induced by the FPR1-selective agonist fMLF, was not influenced by the presence of **8** (Figure 5C inset).

Influence of Fatty Acid Length and Stereochemistry of the Aoc Residue in the Headgroup. Interestingly, while the size of the N-terminal fatty acid moiety was of minor importance for the parent compound **2**, replacement of the N-terminal lauric acid in **5** with the shorter decanoic acid (to give **6**) decreased potency, while a two-carbon increased length of the fatty acid (to give **7**) led to a ∼2-fold increased potency (Table 2). In addition, the structural requirements for agonism/antagonism of analogues of **8**, displaying different lengths of N-terminal fatty acids (i.e., **9** and **10**), were examined, but neither **9** nor **10** induced any release of superoxide in neutrophils, demonstrating their lack of agonistic effects, while they retained a weak FPR2-selective antagonistic profile like the parent **8** (Table 2). The highest antagonist

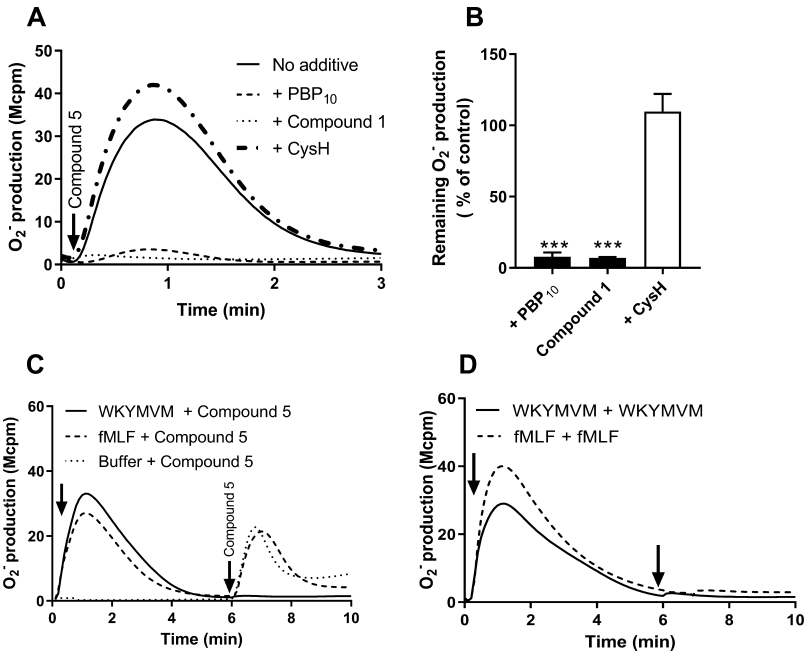

Figure 4. Production of O_2^- in neutrophils induced by compound **5** is mediated via FPR2. (A) Neutrophils (10^5 cells/mL) were preincubated (5 min at 37 °C) with the FPR2-selective inhibitors PBP_{10} (1 μM) or compound **1** (1 μM) or with the FPR1-selective inhibitor CysH (1 μM) prior to stimulation with compound **5** (250 nM), and then the production of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10^6 × counts/min; Mcpm). A representative data set obtained from at least three independent experiments ($n \geq 3$) is shown. (B) Inhibition of production of O_2^- induced by compound **5** in human neutrophils. Neutrophils (10^5 cells/mL) were preincubated (5 min at 37 °C) with or without receptor-selective antagonist (CysH for FPR1, 1 μM; PBP_{10} or compound **1** for FPR2, 1 μM). The cells were then stimulated with compound **5** (250 nM), and then the O_2^- production was measured over time. The results are presented as normalized to the response induced by compound **5** in the absence of an inhibitor (determined as the amount of O_2^- produced; peak value). The statistical significance was calculated with one-way ANOVA using Dunnett's multiple comparisons test against control that received no inhibitor (mean ± SD; $n = 3$), ***$p < 0.001$. (C) Neutrophils (10^5 cells/mL) were prewarmed (5 min at 37 °C) before stimulation with WKYMVM (100 nM) or fMLF (100 nM). When the response had declined to baseline, a subsequent stimulation with compound **5** (250 nM) on the same cells was performed (as indicated by the second arrow). The response from naïve neutrophils treated with compound **5** (buffer control) is shown for comparison. Abscissa: time of study (min); ordinate: superoxide production (10^6 × counts/min; Mcpm). (D) Neutrophils (10^5 cells/mL) were prewarmed (5 min at 37 °C) before stimulation with WKYMVM (100 nM, solid line) or fMLF (100 nM, dashed line). When the responses had declined to baseline, a subsequent stimulation with WKYMVM (100 nM) or fMLF (100 nM) on the same cells was performed (as indicated by the second arrow). A representative data set is shown of at least three independent experiments (i.e., $n \geq 3$).

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

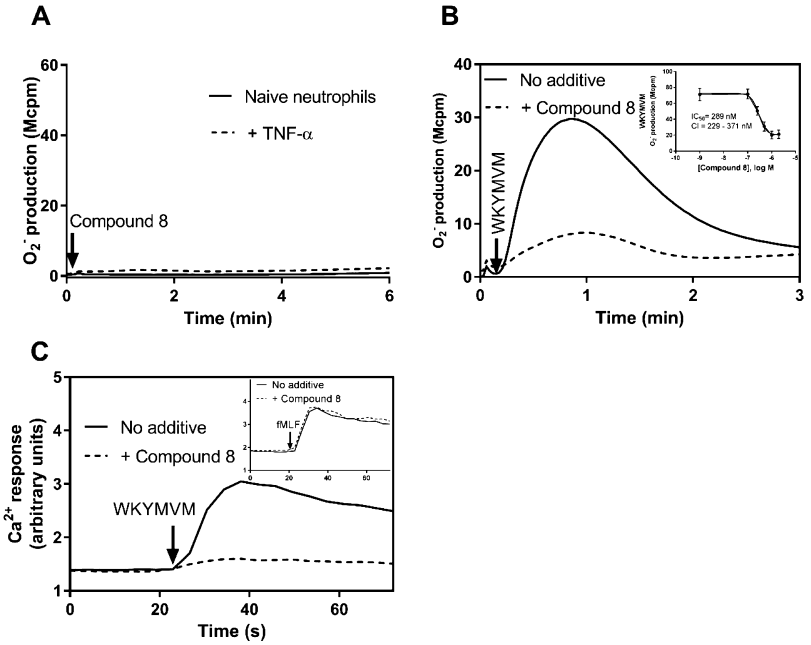

Figure 5. Peptoid backbone stereochemistry is critical for agonist/antagonist activity. (A) Naïve neutrophils (10^5 cells/mL) or neutrophils pretreated with TNF-α (for 20 min at 37 °C) were preincubated (5 min at 37 °C), and were then stimulated with compound **8** (1 μM). Subsequently, the release of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10^6 × counts/min; Mcpm). A representative data set from at least three independent experiments (i.e., $n \geq 3$) is shown. (B) Neutrophils (10^5 cells/mL) were preincubated (5 min at 37 °C) with or without compound **8** (1 μM), and were then stimulated with WKYMVM (100 nM). Subsequently, the release of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10^6 × counts/min; Mcpm). A representative data set from at least three independent experiments (i.e., $n \geq 3$) is shown. The inset shows an inhibitory dose−response curve for compound **8**. Data are presented as the normalized peak response (mean ± SD; $n = 3$), together with the fitted curve and the calculated IC_{50} value (95% CI). (C) The intracellular calcium transient was measured in neutrophils labeled with Fura-2. Cells were preincubated (10 min at 37 °C) with or without compound **8** (1 μM), and were then stimulated with WKYMVM (10 nM), after which the concentration of free intracellular calcium was measured by the Fura-2 fluorescence. Inset: Cells were preincubated (10 min at 37 °C) with or without compound **8** (1 μM), and were then stimulated with fMLF (10 nM). Abscissa: time of study (s); ordinate: fluorescence (arbitrary units). A representative data set from at least three independent experiments (i.e., $n \geq 3$) is shown.

concentration tested was 1 μM, at which inflection of the inhibition curve was not observed. By contrast, our earlier published closely related but strongly antagonistic peptidomimetics (e.g., **1**) had IC_{50} values in the range 50−100 nM.[18] Consequently, the antagonistic analogues, identified in the present work, were considered to display only weakly antagonistic properties. Hence, on the basis of these findings, the stereochemistry of the peptoid units appears to be a most critical determinant of the type of FPR2 interaction displayed by this class of peptidomimetic ligands, but notably FPR2 selectivity is retained for all these peptidomimetic analogues (i.e., **2**−**10**).

The precise function of the N-terminal fatty acid moiety in the FPR2 interaction is not clear, but many lipidated molecules are modulators of FPR2 function, and these include the FPR2-modulating pepducins.[5] For pepducins, suggested to mediate their modulating action on the intracellular signaling domains of the targeted receptor, the fatty acid is considered to enable peptide translocation across the lipid bilayer.[27,28] This concept may well apply to other GPCR-derived pepducins that interact with their respective receptors, but the mode of action of FPR2-activating/inhibiting pepducins appears to be unique because recent results infer that they interact with extracellular receptor parts. This issue has been discussed in detail in earlier publications.[6,16,25,29] This pattern recognition model is supported by the finding that lipidated peptidomimetics also preferentially display affinity for FPR2 on the cell surface.[17,18] Nevertheless, when only an N-terminal fatty acid is present on a peptidomimetic tail or the headgroup (*N*-acylated Aoc) is conjugated to an α-peptide/β-peptoid tail sequence possessing inappropriate chirality of the hydrophobic β-peptoid side chains, the resulting compounds lack agonistic effects on neutrophils, showing that interactions of the peptidomimetic tail sequence also contribute to FPR2 agonism.[17−19]

In line with our previous results for compound **2**, the stereochemistry of the Aoc residue was also of utmost importance for both type and degree of activity. Thus, compounds **11** and **12**, both displaying an (*R*)-Aoc residue instead of (*S*)-Aoc present in **5** (Table 2), were almost devoid of neutrophil-activating effect. On the other hand, for antagonistic analogues, the stereochemistry of the Aoc residue did not substantially affect the activity (i.e., **13** vs **8**, Table 2).

Collectively, these findings suggest that the functional groups displayed by the peptidomimetic tail and the headgroup residues all interact with sites within FPR2, which, depending on the degree of the fit, will result in activation or inhibition. The latter most likely arise from a slight deviation from the optimal orientation of one or more crucial functional groups in an antagonistic ligand that, despite lack of ability to induce the conformational change associated with activation, blocks binding of agonists or locks the receptor in an inactive conformation.

Substitutions Vicinal to the N-Terminal Hydrophobic Headgroup Alter Activity and Potency. The quite specific

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

Table 2. Compounds Tested for FPR2 Interaction by Using the NADPH Oxidase Activation Assay

no.	sequence	EC_{50} nM (CI)	comment	ref
Variation of Fatty Acid and Peptoid Chirality				
2	Lau-[(*S*)-Aoc]-[Lys-*β*NPhe]$_6$-NH_2	167 (142−197)	partial agonist[a,b]	17
3	Dec-[(*S*)-Aoc]-[Lys-*β*NPhe]$_6$-NH_2	⋙ 200	partial agonist[a,b]	17
4	Myr-[(*S*)-Aoc]-[Lys-*β*NPhe]$_6$-NH_2	176 (153−202)	partial agonist[a]	17
5	Lau-[(*S*)-Aoc]-[Lys-*β*Nrpe]$_6$-NH_2	58 (51−66)	full agonist	
6	Dec-[(*S*)-Aoc]-[Lys-*β*Nrpe]$_6$-NH_2	177 (138−226)	partial agonist	
7	Myr-[(*S*)-Aoc]-[Lys-*β*Nrpe]$_6$-NH_2	35 (25−45)	full agonist	
8	Lau-[(*S*)-Aoc]-[Lys-*β*Nspe]$_6$-NH_2		antagonist[c] IC_{50} = 0.29 *μ*M (0.22−0.37)	
9	Dec-[(*S*)-Aoc]-[Lys-*β*Nspe]$_6$-NH_2		weak antagonist[d]	
10	Myr-[(*S*)-Aoc]-[Lys-*β*Nspe]$_6$-NH_2		weak antagonist[d]	
Variation of Aoc Stereochemistry				
11	Lau-[(*R*)-Aoc]-[Lys-*β*NPhe]$_6$-NH_2	too weak[e]	agonist	17
12	Lau-[(*R*)-Aoc]-[Lys-*β*Nrpe]$_6$-NH_2	too weak[e]	agonist	
13	Lau-[(*R*)-Aoc]-[Lys-*β*Nspe]$_6$-NH_2		weak antagonist[d]	
Variation of Peptoid Residue Adjacent to Headgroup				
14	Lau-[(*S*)-Aoc]-Lys-*β*Nrpe-[Lys-*β*NPhe]$_5$-NH_2	29 (22−37)	full agonist	
15	Lau-[(*S*)-Aoc]-Lys-*β*Nspe-[Lys-*β*NPhe]$_5$-NH_2		weak antagonist[d]	
Insertion of Hydrophobic Amino Acid (Instead of *β*-Peptoid) Adjacent to Headgroup				
16	Lau-[(*S*)-Aoc]-Lys-Phe-[Lys-*β*NPhe]$_5$-NH_2	140 (117−163)	full agonist	
17	Lau-[(*S*)-Aoc]-Lys-Tyr-[Lys-*β*NPhe]$_5$-NH_2	49 (45−53)	full agonist	
18	Lau-[(*S*)-Aoc]-Lys-Trp-[Lys-*β*NPhe]$_5$-NH_2	208 (134−321)	partial agonist[a]	
Replacement of Lys Adjacent to Headgroup				
19	Lau-[(*S*)-Aoc]-Dab-*β*NPhe-[Lys-*β*NPhe]$_5$-NH_2		weak antagonist[d]	
20	Lau-[(*S*)-Aoc]-Dab-Phe-[Lys-*β*NPhe]$_5$-NH_2		weak antagonist[d]	
21	Lau-[(*S*)-Aoc]-(D-Lys)-Phe-[Lys-*β*NPhe]$_5$-NH_2		weak antagonist[d]	
22	Lau-[(*S*)-Aoc]-*β*NLys-Phe-[Lys-*β*NPhe]$_5$-NH_2		weak antagonist[d]	
24	Lau-[(*S*)-Aoc]-Nle-Phe-[Lys-*β*NPhe]$_5$-NH_2	87 (76−105)	full agonist FPR2 > FPR1	
25	Lau-[(*S*)-Aoc]-Arg-Phe-[Lys-*β*NPhe]$_5$-NH_2	45 (42−49)	full agonist	
Reversed Backbone				
23	Lau-[(*S*)-Aoc]-(*β*NLys-Phe)$_6$-NH_2		weak antagonist[d]	

[a]Defined as significant lower max response (in O_2^- production) than that induced by WKYMVM (Supporting Information, Figure S1). [b]E_{max} values are provided in ref 17 and in Supporting Information, Figure S1. [c]Determined by the inhibition of the O_2^- production induced by WKYMVM. [d]Inhibitory activity but no IC_{50} could be determined because inflection of the dose−response curve was not achieved at the highest concentration tested (1 *μ*M). [e]Activation of superoxide release was observed, but the inflection point of the dose−response curve was not reached at the maximum concentration tested (1 *μ*M), and thus no EC_{50} value could be determined.

requirements for both the fatty acid and Aoc residues for FPR2 activation infer that the entire N-terminal headgroup is indeed involved in crucial ligand−receptor interactions initiating signaling. In accordance with this, even small changes to the Lau-(*S*)-Aoc headgroup, characteristic of potent peptidomimetic agonists, give rise to analogues with weakened agonistic or even antagonistic properties.[17] In the present study, an additional aim was to investigate whether the residues vicinal to the hydrophobic headgroup constitute potential sites for further optimization of agonist−receptor interactions. Initially, analogues were designed to display variation only in the stereochemistry of the first *β*-peptoid unit (i.e., replacement of the vicinal *β*NPhe in **2** with *β*Nrpe or *β*Nspe to give **14** and **15**, respectively), while retaining achiral *β*NPhe units in the C-terminal region of the peptidomimetic tail. Compared to **5**, analogue **14** retained full activation potential, and its potency was higher (Table 2 and Figure 6). Importantly, **14** retained FPR2 preference as shown by the fact that the FPR2-selective antagonist PBP_{10} but not the FPR1-specific antagonist cyclosporin H inhibited its activity (as shown for PBP_{10} in Figure 6A). The response was of the same order of magnitude as that of the FPR2 agonist WKYMVM (Figure 6B), but in contrast to WKYMVM, the neutrophil-activating effect of compound **14** was not affected by myeloperoxidase (MPO)-derived reactive oxygen species (Figure 6C). Peptidomimetic **14** thus shows better oxidative stability than the conventional peptide agonist.

These data indeed infer that interactions with several functional groups in the N-terminal part of the peptidomimetic tail region are involved in mediating FPR2 activation. In support of this hypothesis, introduction of a single *β*Nspe in this position (to give **15**) proved sufficient to abolish its capability for FPR2 activation. Nonetheless, the ability of **15** to inhibit the WKYMVM response was lowered (Table 2), suggesting that other groups contribute more to the propensity for inhibition.

Thus, the data obtained so far indicate the importance of functional groups within the N-terminal headgroup and in the residues adjacent to this (i.e., Lau-[(*S*)-Aoc]-Lys-X; with X = *β*Nrpe conferring highest potency) in the activation of FPR2 by such peptidomimetics. In order to investigate the nature of these interactions further, we prepared a series of variants of **2**, in which the lysine and/or the *β*NPhe residues in the extended headgroup were replaced by *α*-amino acids with different but related functional groups. Replacement of the first *β*NPhe in **2** with the corresponding *α*-amino acid Phe (resulting in **16**) had no significant effect on the relative potencies [EC_{50} of 167 nM (95% CI: 142−197) and 140 nM (95% CI: 117−163) for **2** and **16**, respectively]. In contrast, similar replacement with Tyr (to give **17**) further increased the potency [to an EC_{50} of 49 nM (95% CI: 45−53)], while substitution with Trp (to give **18**) led only to a slightly reduced potency [EC_{50} of 208 nM (95% CI: 134−321); Table 2]. These data suggest that the hydrophobic functional group in the side chain of the residue in this position is involved in the receptor interaction

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

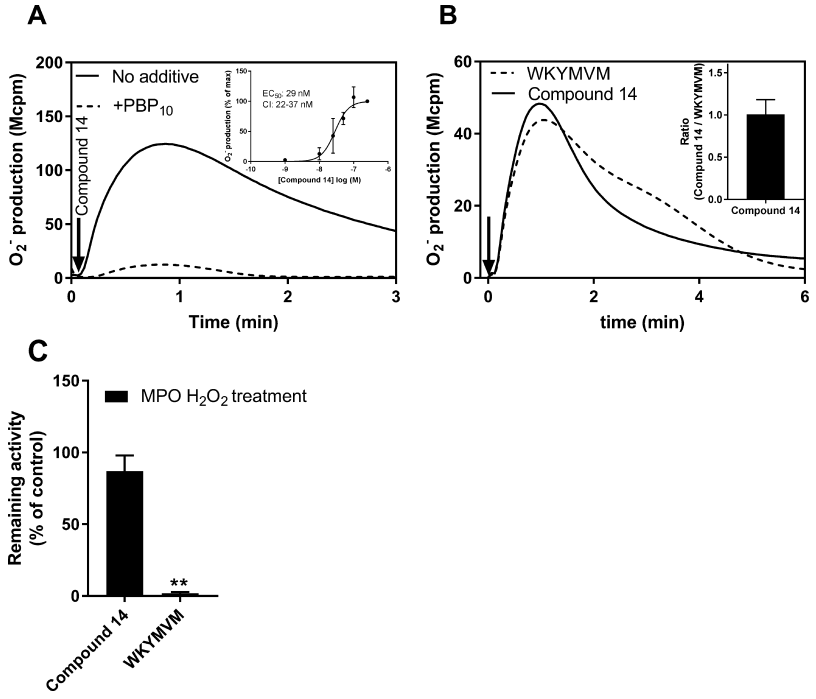

Figure 6. (A) Compound **14** is a highly potent FPR2-activating peptidomimetic. Neutrophils (10^5 cells/mL) were preincubated (5 min at 37 °C) with the FPR2-selective inhibitor PBP_{10} (1 μM) or buffer before stimulation with compound **14** (100 nM), and then the production of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10^6 × counts/min; Mcpm). A representative data set out of three independent experiments (n = 3) is shown. Inset: Dose-dependent superoxide release induced by compound **14**; data are presented as normalized peak response (mean ± SD; n = 3), and the fitted curve as well as the EC_{50} value and 95% confidence interval are calculated. (B) Neutrophils (10^5 cells/mL) were preincubated for 5 min at 37 °C, and were then stimulated with compound **14** (1 μM) or WKYMVM (100 nM), and then release of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10^6 × counts/min; Mcpm). A representative data set out of three independent experiments (n = 3) is shown. Inset: Comparison of the responses induced by compound **14** and WKYMVM is presented as the ratio between the peak values obtained with compound **14** and WKYMVM, respectively (mean ± SD; n = 3). (C) Compound **14** is not sensitive to oxidation by the MPO−H_2O_2 system. The peptide WKYMVM (1 μM) and compound **14** (1 μM) were incubated with MPO (1 μg/mL) and H_2O_2 (10 μM). The remaining activity of the agonists (WKYMVM and compound **14** after 100× dilution in the oxidase-measuring system) was determined by their ability to activate the neutrophil NADPH oxidase. Neutrophils (10^5 cells/mL) were activated with untreated and MPO−H_2O_2-treated compound **14** (100 nM final concentration) or WKYMVM (100 nM final concentration), and then the peaks of the responses were determined. The remaining activation potencies are presented as the percentage relative to the responses induced by the untreated agonists (controls) and treated agonists (mean ± SD; n = 3). Statistical analysis was performed by using paired Student's t-test, $^{**}p < 0.01$).

promoting activation, albeit some polarity appears to be preferable in this position.

Furthermore, replacement of the N-terminal lysine in **2** or **16** with (*S*)-2,3-diaminobutanoic acid (Dab), having a two-carbon shorter side chain (to give **19** or **20**), abrogated all FPR activation when the adjacent hydrophobic residue was Phe or βNPhe; however, the resulting analogues exhibited weak antagonistic properties. Likewise, replacement of this Lys with D-Lys or βNLys, conferring altered or absence of chirality (in the resulting **21** or **22**), respectively, also led to a loss of agonistic activity. However, in line with previous minor modifications of **2**, these molecules possessed inhibitor/antagonistic properties, implying that the cationic Lys adjacent to Aoc is also directly involved in receptor interactions. Moreover, analogue **23** displaying a completely reversed design with alternating cationic β-peptoid residues (i.e., βNLys units) and hydrophobic Phe residues proved to possess only weak FPR2-antagonistic properties. In compound **24**, the first Lys was replaced by the noncharged Nle, which increased its potency almost 2-fold as compared to that of **16**. Interestingly, the receptor selectivity of **24** was reduced, as it activated both FPR1 and FPR2. This was evident from the observation that in order to achieve full inhibition of the response induced by **24**, the antagonists CysH (FPR1-selective) and PBP_{10} (FPR2-selective) had to be employed in combination (Supporting Information, Figure S2). Analogue **25**, displaying a Lys → Arg substitution (and thus a slightly elongated cationic side chain), possessed a ∼3-fold increased potency as compared to that of **16**, while retaining FPR2 selectivity (Table 2).

These structure−activity relationships indicate that chirality as well as length and charge of the side chain in the residue adjacent to Aoc influences the ability of the analogue to activate FPRs. Noticeably, charge determines whether the analogue exhibits full selectivity for FPR2 over FPR1. These findings correspond well with recent results obtained in a combined mutagenesis/computer modeling study, showing that the primary difference between FPR1 and FPR2, regarding their respective orthosteric binding sites for formyl peptides, relates to their electrostatic potential: the binding site in FPR2 is negatively charged because of the presence of an Asp in position 281, whereas FPR1 contains a neutral Gly in the same position.[30]

Pronounced FPR2 Reactivation via Cross-Talk with the Receptors for PAF and ATP in Peptidomimetic-Desensitized Neutrophils. During infection/inflammation, microbes or damaged host cells release several danger

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

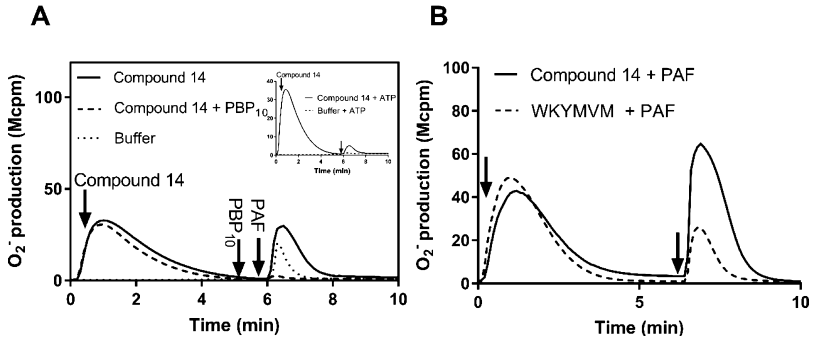

Figure 7. Neutrophils desensitized with compound **14** can be reactivated by PAF to a greater extent than neutrophils desensitized with WKYMVM. (A) Neutrophils (10^5 cells/mL) were first desensitized with compound **14** (250 nM) and subsequently stimulated with PAF (100 nM; as indicated by the arrow: solid line), and then release of superoxide anions was recorded continuously. In one sample, the FPR2-selective inhibitor PBP_{10} (1 μM) was added 1 min prior to PAF stimulation (dashed line). The PAF responses induced in naïve cells (dotted line) are shown for comparison. A representative data set out of three independent experiments (n = 3) is shown. Inset: Neutrophils (10^5 cells/mL) were first desensitized with compound **14** (250 nM) and subsequently stimulated with ATP (50 μM; as indicated by the arrow: solid line). The ATP (50 μM) responses induced in naïve cells (dashed line) are shown for comparison. Abscissa: time of study (min); ordinate: superoxide production (10^6 × counts/min; Mcpm). (B) Neutrophils (10^5 cells/mL) were first desensitized with compound **14** (250 nM; solid line) or WKYMVM (100 nM; dashed line) and subsequently stimulated with PAF (100 nM; as indicated by the arrow), and then release of superoxide anions was recorded continuously. Abscissa: time of study (min); ordinate: superoxide production (10^6 × counts/min; Mcpm). A representative data set out of three independent experiments (n = 3) is shown.

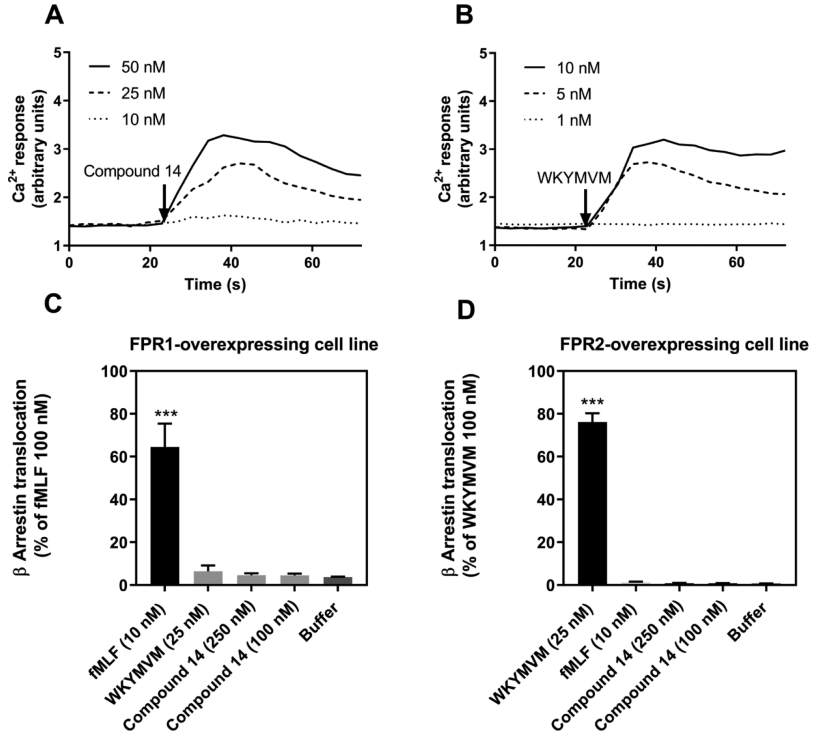

Figure 8. Compound **14** dose-dependently induces an intracellular calcium transient but no β-arrestin recruitment. (A) Intracellular calcium transient was measured in neutrophils loaded with Fura-2. Cells were preincubated (10 min at 37 °C) and then stimulated with compound **14** in different concentrations (50, 25, and 10 nM) or (B) with WKYMVM (10, 5, and 1 nM), after which the concentration of free intracellular calcium was measured by the Fura-2 fluorescence. Abscissa: time of study (s); ordinate: fluorescence (arbitrary units). Representative data sets out of three independent experiments (n = 3) are shown in A and B. (C/D) FPR2-induced β-arrestin translocation was measured in PathHunter CHO cells from DiscoverX that coexpressed prolinked FPR1/FPR2 and β-galactosidase. Binding of β-arrestin was quantified by fluorescence through enzyme complementation. The prototypic FPR1 agonist fMLF (100 and 10 nM) induced β-arrestin translocation in FPR1-overexpressing cells, whereas the FPR2 agonist WKYMVM (100 and 25 nM) induced β-arrestin translocation in FPR2-overexpressing cells. In contrast, peptidomimetic **14** (at 250 and 100 nM) failed to induce any β-arrestin translocation in both cell lines. Data are expressed as mean ± SD from three independent experiments (i.e., n = 3). The statistical significance was calculated with one-way ANOVA using Dunnett's multiple comparison test against the buffer control. $^{**}p < 0.001$.

molecules/chemoattractants, including formyl peptides that are sensed by neutrophils through surface-expressed GPCRs.[6] These receptors cross-talk through different mechanisms and together they regulate neutrophil directional migration and other effector responses.[31,32] Previously, it was found that upon desensitization FPR2 may be reactivated by receptor cross-talk

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

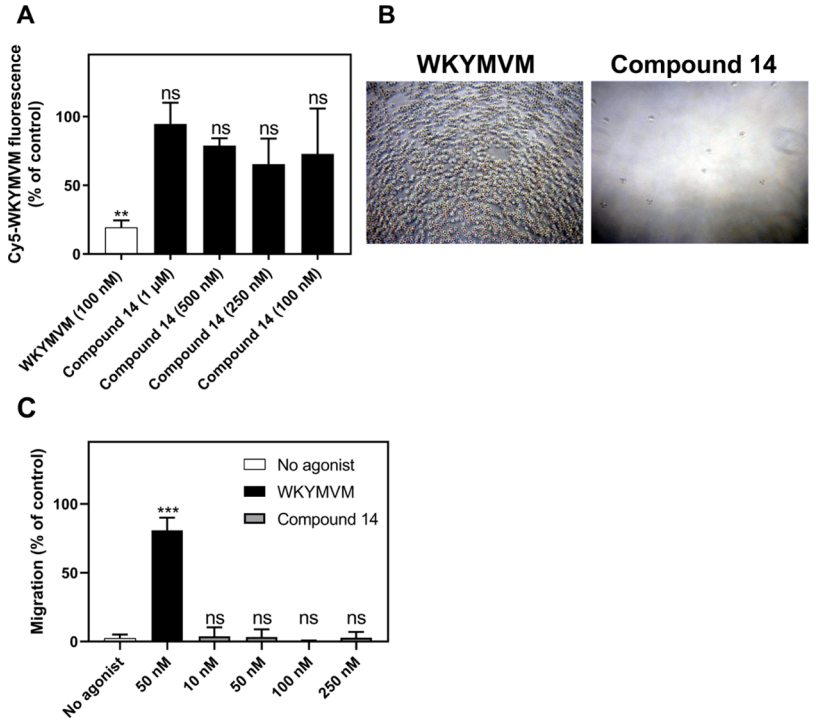

Figure 9. Compound **14** neither competes with the FPR2 agonist WKYMVM for binding nor induces neutrophil migration. (A) No competitive inhibition of binding of the conventional FPR2 agonist WKYMVM is mediated by compound **14**. Neutrophils (10^6 cells/mL) were incubated (10 min on ice) without any additive (control to determine total binding) and with WKYMVM (100 nM) or compound **14** (100 nM−1 μM), after which the fluorescently labeled FPR2-selective agonist Cy5-WKYMVM (1 nM) was added, and then incubation was continued for 60 min. The results showed remaining fluorescence activity in the presence of nonlabeled WKYMVM or compound **14** as compared to total binding expressed in percent of the value for the control (mean ± SD, n = 3−5). As determined by a one-way ANOVA using Dunnett's multiple comparison test, no significant inhibition was obtained with compound **14** as compared to the control (total binding); ns: not significant, $^{**}p < 0.01$. (B) Representative microscopy images of neutrophils recovered in the lower compartment of the chemotactic chamber with WKYMVM (50 nM; left) or compound **14** (100 nM; right) present below the filter. (C) Number of neutrophils recovered in the lower compartment with WKYMVM (50 nM) or different concentrations of compound **14** (10−250 nM) is expressed in percent of the number of neutrophils in response to the FPR1 agonist fMLF (10 nM). The number of neutrophils recovered in the absence of any agonist (spontaneous migration) is also shown for comparison (mean ± SD, n = 3). As determined by a one-way ANOVA using Dunnett's multiple comparison test, no significant increase in migration was obtained with compound **14** as compared to no agonist; ns: not significant, $^{***}p < 0.001$.

induced by ATP or PAF upon binding to their respective receptors. The molecular origin of this cross-talk reactivation is not clear, but we have shown that the reactivation signaling pathway bypasses the PLC-PIP_2-Ca^{2+} signal generated downstream of the receptors.[33] To determine the ability of PAF and ATP to reactivate peptidomimetic-desensitized FPR2 (for more details about the receptor cross-talk reactivation process, see refs[32−36]), neutrophils desensitized with **14** were subjected to reactivation through addition of PAF (an agonist for PAFR) or ATP (an agonist for $P2Y_2R$). The PAF-induced response was primed in comparison to the response induced in naïve cells (Figure 7A). In agreement with a cross-talk mechanism for reactivation of FPR2, the PAF-induced response proved sensitive to the FPR2-selective antagonist PBP_{10} when this was added prior to stimulation with PAF (Figure 7A). A very similar reactivation pattern was observed when PAF was replaced with ATP that activates the neutrophil $P2Y_2R$ (Figure 7A inset). More importantly, when the cross-talk reactivation in neutrophils desensitized with **14** was compared to that observed for cells desensitized with the conventional peptide FPR2 agonist WKYMVM, the response was substantially higher in cells desensitized with **14** (Figure 7B). A higher concentration of WKYMVM proved not to compensate for the increased potency of **14** in cross-talk reactivation. We have shown earlier that PAF/ATP is also capable of potent reactivation of neutrophils desensitized with FPR2-selective lipopeptides belonging to the pepducin family of GPCR-activating agonists,[34,35] suggesting similarities between the signaling induced by FPR2-selective pepducins and lipidated peptidomimetics.

FPR2-Selective Peptidomimetics Trigger the Calcium Signaling Pathway without Recruitment of β-Arrestin. Signaling induced by the FPR2-selective neutrophil-activating pepducin ($F2Pal_{10}$) was recently shown to be biased toward activation of the PLC-PIP_2-Ca^{2+} pathway, while β-arrestin was not recruited.[16] The β-arrestin signaling pathway cannot be quantitatively determined in neutrophils, and therefore, the PathHunter enzyme fragment (EA) complementation technology in FPR-overexpressing CHO cells was used to compare the signaling properties of **14** with those of the conventional FPR2-selective agonist WKYMVM.

At concentrations of 25 and 50 nM, compound **14** evoked a transient increase in intracellular Ca^{2+} level in neutrophils (Figure 8A), whereas WKYMVM at concentrations of 5 and 10 nM induced a similar concentration-dependent increased Ca^{2+} level (Figure 8B). Expectedly, fMLF (at 10 nM) only triggered β-arrestin recruitment in FPR1-overexpressing cells because of its FPR1 selectivity (Figure 8C,D), while neither WKYMVM nor compound **14** affected these cells (Figure 8C). From the β-arrestin translocation experiments with FPR2-

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

overexpressing cells, WKYMVM (at 25 nM) proved capable of β-arrestin recruitment, suggesting that FPR2 is properly expressed, and that the system to recruit β-arrestin upon stimulation is intact in these cells (Figure 8D). In contrast, compound **14** failed to induce β-arrestin recruitment even at concentrations that induced maximal superoxide release; however, this was not due to adverse effects on the cells caused by the peptidomimetics because the cells treated with **14** proved to be viable (see Supporting Information, Figure S3).

Overall these findings demonstrate that both WKYMVM (a conventional FPR2 agonist) and the FPR2-activating peptidomimetics trigger a G-protein-dependent Ca^{2+} response, whereas only binding of WKYMVM to FPR2 results in β-arrestin recruitment. Thus, the peptidomimetic agonists (e.g., **2**, **5**, and **14**) exhibited biased FPR2 signaling (see Supporting Information, Figure S1), and thus, these compounds share this signaling property with FPR2-activating pepducins such as $F2Pal_{10}$.[16] Interestingly, biased FPR agonism has been proposed as a new approach in the therapy for myocardial injury.[37]

Notably, despite the fact that β-arrestin was not recruited by FPR2 when triggered by these peptidomimetic agonists, the activated receptor is rapidly desensitized after activation (Figure 4). This is in line with reports showing that FPR desensitization is achieved primarily through a cytoskeleton-dependent process in which polymerized actin replaces β-arrestin, thereby segregating the agonist-bound receptor from the signaling G-protein.[16,38,39] Even though the precise signals involved in receptor cross-talk remain unknown, we show that neutrophils desensitized with the lipidated peptidomimetic **14** can be reactivated to produce substantial amounts of superoxide anions when PAFR or $P2Y_2R$ is triggered by PAF or ATP, respectively.

Peptidomimetic 14 Is Not a Chemoattractant and Does Not Compete in Binding with the FPR2-Selective Agonist WKYMVM. To further elucidate key features regarding the mechanism for neutrophil activation/desensitization/reactivation induced by **14**, binding experiments were performed by using a fluorescently labeled peptide agonist for FPR2. As expected, nonlabeled WKYMVM in excess (i.e., the ratio between nonlabeled and labeled peptide was 100:1) reduced Cy5-WKYMVM binding significantly (Figure 9A), whereas no significant reduction of binding was obtained with **14** in concentrations up to 1 μM (i.e., nonlabeled **14** to Cy5-WKYMVM in the ratio 1000:1; Figure 9A). These findings corroborate previously reported data, inferring that binding of the fluorophore-labeled FPR2-selective Cy5-WKYMVM ligand cannot be competitively displaced by compound **2**.[17] We have previously identified $F2Pal_{10}$ as a biased FPR2 agonist, which lacks the capacity to recruit β-arrestin and is devoid of chemotactic properties.[16] To investigate whether compound **14** possessed the same activation profile, the ability of compound **14** to trigger a chemotactic response in neutrophils was determined. In contrast to WKYMVM (a positive control), compound **14** did not induce any chemotactic migration (Figure 9B,C).

Clearly, FPR2 has a broad ligand recognition profile, as it displays high affinity for the microbial/mitochondrial formyl peptide molecular pattern, but, on the other hand, it is promiscuous as it accommodates binding of several other types of ligands that may activate the receptor or inhibit receptor function. Although the precise binding pocket for the well-characterized FPR2 ligands has not yet been clearly defined, it is now apparent that biased agonists may stabilize receptors in conformations different from that triggered by conventional agonists. Hence, these different subactive conformations appear to trigger distinct sets of signaling modes (e.g., G-protein-dependent and/or β-arrestin-dependent), leading to selective induction of certain cellular responses.[10,15,40] The present data indicate that WKYMVM and peptidomimetic ligands (such as **14**) may utilize different binding sites and that the peptidomimetic ligands may act as allosteric agonists. Furthermore, this difference in binding-site occupancy may contribute to the characteristic FPR2-mediated signaling properties exerted by each of these ligand types.

Indeed, it would be interesting to compare the in vivo effects of biased and nonbiased agonists in models of infectious and/or inflammatory diseases to delineate the contribution of these different FPR-dependent signaling pathways and cellular responses to induction and resolution of inflammation, and thus the therapeutic potential of targeting these pathways. However, for such a comparison to be accurate, biased and nonbiased agonists should preferably belong to the same compound type to ensure similar biodistribution and stability. Thus, future development of non-biased FPR2-selective agonists belonging to the present class of peptidomimetics would allow for a delineation of the molecular requirements for induction of β-arrestin recruitment and chemotaxis, which both are highly relevant signaling properties.

CONCLUSIONS

Regarding FPR2 activation and inhibition by lipidated α-peptide/β-peptoid hybrids, the present study provides structural insights into the critical role of the two residues vicinal to the N-terminal hydrophobic headgroup as well as the effects of presence/absence of α-chirality in the peptoid residues of the tail region. These peptidomimetics constitute a lipidated subclass of α-peptide/β-peptoid hybrids, which earlier were found to resist proteolytic degradation by generalist enzymes such as pronase and specific enzymes such as trypsin and chymotrypsin.[8,9] Also, these FPR2-selective agonists were found to exert biased signaling and mediate functional selectivity in human neutrophils. Nevertheless, further studies are required to elucidate the exact role of β-arrestin in signaling, causing a reactivation of desensitized FPRs. In summary, these proteolytically stable peptidomimetics with unique signaling properties may constitute excellent tools for elucidating the mechanistic details in FPR2-mediated signaling as well as for disclosing its immunoregulatory function in vivo. In addition, future studies should also increase our understanding of the impact of these novel FPR2 ligands on other cells that endogenously express FPR2.

EXPERIMENTAL SECTION

Materials. Solvents, Fmoc-protected amino acid building blocks, Rink amide resin, and coupling reagents were purchased from IrisBiotech (Marktredwitz, Germany), while Lau-OSu, decanoic acid, and myristic acid were from Sigma-Aldrich Chemie (Steinheim, Germany). Dextran and Ficoll–Paque were obtained from GE-Healthcare Bio-Science (Uppsala, Sweden). Horseradish peroxidase (HRP) was obtained from Boehringer Mannheim (Germany). The hexapeptide WKYMVM was obtained from AltaBioscience

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

(University of Birmingham, Birmingham, UK), and the PIP_2-binding peptide PBP_{10} was obtained from Caslo Laboratory (Lyngby, Denmark). Isoluminol, fMLF, bovine serum albumin (BSA), TNF-α, and ATPγS were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Fura-2 was from Molecular Probes, while Cy5-WKYMVM was from Phonenix Pharmaceutical (Burlingame, CA). Cyclosporin H (CysH) was kindly provided as a gift from Novartis Pharma (Basel, Switzerland), while PAF was obtained from Avanti Polar Lipids Inc. (Alabama, USA). Peptide stock solutions were prepared in dimethyl sulfoxide and stored at −70 °C until use. Subsequently, all dilutions of reagents were made with Krebs−Ringer phosphate buffer (KRG, pH 7.3; 120 mM NaCl, 5 mM KCl, 1.7 mM KH_2PO_4, 8.3 mM NaH_2PO_4, and 10 mM glucose) supplemented with Ca^{2+} (1 mM) and Mg^{2+} (1.5 mM).

General Methods for Compound Characterization. The identity of compounds was determined by high-resolution mass spectrometry, while purity was measured by ultrahigh-performance liquid chromatography (UHPLC) to be at least 97% (detection at λ = 220 nm). Analytical UHPLC was performed on a Shimadzu Prominence UHPLC system by using a Phenomenex Luna C18(2) HTS column (100 × 3.0 mm; particle size: 2.5 μm), eluted at a rate of 0.5 mL/min. Injection volumes were 5−10 μL of ∼1 mg/mL solutions. Separations were performed at 40 °C. Eluents: A [H_2O/acetonitrile (MeCN)/trifluoroacetic acid (TFA) 95:5:0.1] and B (MeCN/H_2O/TFA 95:5:0.1) were used for all peptidomimetics in a linear gradient of 20% B → 80% B during 10 min. Separation by preparative HPLC was performed on a Phenomenex Luna C18(2) column (250 × 21.2 mm; particle size: 5 μm) on a Shimadzu system composed of a CBM-20A Prominence communication bus module, two LC-20AP Prominence pumps, an SPD-M20A Prominence diode array detector, and an SIL-20A HT Prominence autosampler. The above eluents A and B were employed with a flow rate of 20 mL/min; injection volumes were 300−900 μL, and a linear gradient of 20% B → 80% B during 20 min was employed. High-resolution MALDI-TOF spectra were obtained on a Bruker SolariX XR in MALDI mode giving (m/z): $[M + H]^+$ for all peptidomimetics with ΔM below 5 ppm.

Synthesis of Peptidomimetics. Peptidomimetics **5**−**10** and **12**−**25** were prepared by using standard Fmoc-based solid-phase synthesis in Teflon reactors (10 mL): a Rink amide resin (loading, 0.5−0.7 mmol/g; 0.05−0.1 mmol scale) and corresponding dimeric building blocks were employed.[17,41] Removal of Fmoc protecting groups was performed with 20% piperidine in *N*,*N*-dimethylformamide (DMF, 2 × 10 min, each with 5 mL, including a 1 min wash with DMF in between). After the third coupling and henceforth, Fmoc removal was performed in the following way: 20% piperidine in DMF (2 × 5 min, each with 5 mL; with a 1 min DMF wash in between) and 2% DBU +2% piperidine in DMF (2 × 5 min, each with 5 mL; with 1 min DMF wash in between). Upon completing each coupling, the resin was washed with DMF, MeOH, and dichloromethane (DCM, 3 × 2 min, each with 5 mL), and after Fmoc deprotection, it was washed with DMF, MeOH, and DCM (3 × 3 min, each with 5 mL). The building blocks (3 equiv) were coupled by using PyBOP (3 equiv) and diisopropylethylamine (DIPEA, 6 equiv) in DMF (1.5−3 mL) under shaking at room temperature for at least 2 h. Upon the fourth coupling and henceforth, capping with Ac_2O/DIPEA/NMP 1:2:3 (5 mL, 10 min) was applied. After assembly of the peptidomimetic tail region, the N-terminus was functionalized via coupling of Fmoc-Aoc-OH (5 equiv) for 16 h. After the last Fmoc removal, acylation was performed with decanoic acid or myristic acid with PyBOP (5 equiv) as the coupling reagent or via coupling with Lau-OSu (5 equiv, 16 h; 5 equiv DIPEA). Finally, the peptidomimetics were cleaved from the resin by using TFA−water 95:5 (5 mL, 1 h at room temperature). Following evaporation in vacuo, the resulting residues were purified by preparative HPLC. Upon lyophilization, the target compounds were stored at −20 °C until use.

Peptidomimetic ***5***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.29 min. MALDI−TOF: calcd for $[M + H]^+$, 2161.4873; found, 2161.4882; ΔM = 0.4 ppm.

Peptidomimetic ***6***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 5.71 min. MALDI−TOF: calcd for $[M + H]^+$, 2133.4560; found, 2133.4532; ΔM = 1.3 ppm.

Peptidomimetic ***7***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.94 min. MALDI−TOF: calcd for $[M + H]^+$, 2189.5195; found 2189.5167; ΔM = 1.2 ppm.

Peptidomimetic ***8***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.46 min. MALDI−TOF: calcd for $[M + H]^+$, 2161.4873; found 2161.4860; ΔM = 0.6 ppm.

Peptidomimetic ***9***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 5.86 min. MALDI−TOF: calcd for $[M + H]^+$, 2133.4560; found 2133.4564; ΔM = 0.1 ppm.

Peptidomimetic ***10***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 7.07 min. MALDI−TOF: calcd for $[M + H]^+$, 2189.5187; found 2189.5183; ΔM = 0.1 ppm.

Peptidomimetic ***12***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.31 min. MALDI−TOF: calcd for $[M + H]^+$, 2161.4873; found 2161.4913; ΔM = 1.8 ppm.

Peptidomimetic ***13***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.45 min. MALDI−TOF: calcd for $[M + H]^+$, 2161.4873; found 2161.4843; ΔM = 1.3 ppm.

Peptidomimetic ***14***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.13 min. MALDI−TOF: calcd for $[M + H]^+$, 2091.4091; found, 2091.4091; ΔM = 0.0 ppm.

Peptidomimetic ***15***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.21 min. MALDI−TOF: calcd for $[M + H]^+$, 2091.4091; found, 2161.4193; ΔM = 4.8 ppm.

Peptidomimetic ***16***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.13 min. MALDI−TOF: calcd for $[M + H]^+$, 2163.3778; found, 2163.3763; ΔM = 0.6 ppm.

Peptidomimetic ***17***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 5.79 min. MALDI−TOF: calcd for $[M + H]^+$, 2079.3727; found, 2079.3722; ΔM = 0.2 ppm.

Peptidomimetic ***18***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.15 min. MALDI−TOF: calcd for $[M + H]^+$, 2102.3887; found, 2102.3873; ΔM = 0.6 ppm.

Peptidomimetic ***19***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.23 min. MALDI−TOF: calcd for $[M + H]^+$, 2049.3621; found, 2049.3603; ΔM = 0.8 ppm.

Peptidomimetic ***20***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.27 min. MALDI−TOF:

DOI: 10.1021/acsomega.9b00098
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calcd for $[M + H]^+$, 2035.3465; found, 2035.3471; ΔM = 0.2 ppm.

Peptidomimetic ***21***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 5.96 min. MALDI−TOF: calcd for $[M + H]^+$, 2063.3778; found, 2063.3779; ΔM = 0.1 ppm.

Peptidomimetic ***22***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.05 min. MALDI−TOF: calcd for $[M + H]^+$, 2077.3934; found, 2077.3919; ΔM = 0.7 ppm.

Peptidomimetic ***23***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.02 min. MALDI−TOF: calcd for $[M + H]^+$, 2077.3934; found, 2077.3940; ΔM = 0.2 ppm.

Peptidomimetic ***24***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 7.44 min. MALDI−TOF: calcd for $[M + H]^+$, 2048.3669; found, 2048.3690; ΔM = 1.0 ppm.

Peptidomimetic ***25***. Gradient: 20 → 80% B over 10 min. B = 95% MeCN + 0.1% TFA. t_R = 6.25 min. MALDI−TOF: calcd for $[M + H]^+$, 2091.3839; found, 2091.3843; ΔM = 0.1 ppm.

Ethics Statement. In the present study, conducted at the Sahlgrenska Academy, Gothenburg, Sweden, we used buffy coat preparations that were obtained from the blood bank at Sahlgrenska University Hospital, Gothenburg, Sweden. According to Swedish legislation section code 4§ 3p SFS 2003:460 (Lag om etikprövning av forskning som avser människor), no ethical approval was required because the buffy coats were provided anonymously and thus could not be traced back to a specific donor.

Isolation of Human Neutrophils. Peripheral blood neutrophils were isolated from human buffy coats from healthy blood donors by using a dextran sedimentation and Ficoll/Paque gradient centrifugation: the cell-rich plasma/buffy coat was mixed with physiological saline containing dextran (2% w/v; MW 500 000), and then sedimentation of erythrocytes was allowed to take place for 30 min at ambient temperature. The leucocyte-enriched plasma/buffy coat was layered onto a Ficoll/Paque solution (density of 1.077 g/mL) and centrifuged for 15 min (930*g*; 4 °C).[42] The remaining erythrocytes in the neutrophil pellet were subjected to hypotonic lysis, and then upon washing, the neutrophils were resuspended in KRG and stored on melting ice until use. This isolation procedure allows for cell purification with minimal granule mobilization.

Neutrophil NADPH Oxidase Activity. NADPH oxidase activity was measured in an isoluminol-enhanced chemiluminescence (CL) assay: neutrophils were added to a 4 mL polypropylene tube containing isoluminol (2×10^{-5} M) and HRP (2U) in KRG (final volume 900 μL).[43,44] The tubes were subjected to equilibration in the Biolumat for 5 min at 37 °C, after which the stimulating agent (100 μL) was added, and then light emission was recorded continuously. Receptor reactivation and cross-talk were achieved by initial stimulation of cells by exposure to a receptor-specific agonist, and when the response returned to baseline (i.e., the desensitized state), the cells were reactivated by receiving a second stimulation to induce release of superoxide. In these reactivation experiments, the antagonists were added to the CL assay mixture 1 min prior to the second stimulation. The CL response was measured with a six-channel Biolumat LB 9505 (Berthold Co., Germany).

β-Arrestin Recruitment Assay. The capability of FPR2 agonists to promote recruitment of β-arrestin was evaluated in PathHunter eXpress CHO-K1 FPR2-overexpressing cells from DiscoverX (Fremont, USA). The CHO-K1 cells coexpress β-arrestin (isoform 2) tagged with an EA of β-galactosidase and FPR2 tagged with the complementary part (PK) of the β-galactosidase enzyme. Thus, activation of FPR2-PK induces β-arrestin-EA recruitment, forcing complementation of the two β-galactosidase EAs, leading to the assembly of a functional enzyme capable of hydrolyzing the substrate, thereby generating a CL signal. The CHO-K1 cells were seeded into 96-well plates at a density of 10 000 cells/well, which were then grown for 24 h at 37 °C in 5% CO_2. The overnight-cultured cells were activated with agonists and allowed to recruit β-arrestin for 90 min at 37 °C, after which a CL-based detection solution was added (DiscoverX), and then enzyme activity was determined. Light generation, given as relative CL units, was measured with a Multilabel Microtiter Plate Reader (Clariostar, BMG Labtech, Germany).[16]

Calcium Mobilization. Freshly isolated neutrophils in KRG containing 0.1% BSA (5×10^7 cells/mL) were exposed to 5 μM FURA 2-AM for 30 min in the dark at room temperature according to the supplier's protocol (ThermoFisher, USA). The cell suspensions were then diluted 1:2 with RPMI 1640 culture medium without phenol red (PAA Laboratories GmbH, Pasching, Austria), and the resulting suspensions were then centrifuged. Next, the cells were washed once with KRG and were resuspended in KRG to yield a density of 2×10^7 cells/mL. The FURA 2-labeled neutrophils were added to a polystyrene cuvette that was equilibrated in the fluorometer for 10 min at 37 °C, upon which the stimulatory agent (100 μL) was added, and then fluorescence was recorded continuously. These measurements were performed with a PerkinElmer fluorescence spectrophotometer (LC50) having excitation wavelengths set to 340 and 380 nm, an emission wavelength set to 509 nm, and slit widths of 5 and 10 nm, respectively. The transient increase in the intracellular Ca^{2+} level is presented as the ratio between the fluorescence intensities detected (i.e., 340 nm: 380 nm).

Evaluation of Ligand−Receptor Binding by Flow Cytometry. Neutrophils suspended in ice-cold KRG (1×10^6 cells/mL) were preincubated with unlabeled ligands for 10 min while being kept on ice. A fluorophore-labeled FPR2-selective agonist (Cy5-WKYMWM; 1 nM final concentration) was added, and then incubation on ice was continued for an additional 60 min. Binding of the fluorescent FPR2 agonist to the neutrophils was then analyzed by flow cytometry by using the settings for the Cy5 fluorophore (Ex 488 nm/Em 670 nm). For each sample, 10 000 cells were collected by using an Accuri C6 flow cytometer (Becton Dickinson Sparks, MD, USA). Neutrophil samples incubated with Cy5-WKYMVM, either alone or in combination with WKYMVM (100 nM final concentration), were included as controls to determine total binding (Cy5-WKYMVM alone) and nonspecific binding (labeled and unlabeled WKYMVM together), respectively.

Stability of Receptor Ligands to MPO−H_2O_2-Mediated Oxidation. In order to investigate the sensitivity of the FPR2 agonists WKYMVM and peptidomimetic **14** to MPO−H_2O_2-mediated oxidation, the agonists (1 μM concentration) were incubated with MPO (1 μg/mL; 5 min 37 °C) prior to addition of H_2O_2 (final concentration, 10 μM). The samples were incubated for an additional 10 min at room temperature. Agonists incubated under the same conditions, but without

DOI: 10.1021/acsomega.9b00098
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MPO and H_2O_2, were used as controls. The remaining activity of the agonists (WKYMVM and **14**) was determined by their ability to induce neutrophil NADPH oxidase activity as described above.

Neutrophil Chemotaxis. Neutrophil chemotaxis was determined in a filter assay system (ChemoTx, Neuro probe, UK) used essentially as recommended by the manufacturer. In short, neutrophils suspended in KRG supplemented with BSA (0.3% v/w) were added on top of the filter (pore size: 3 μm), and then cell migration was allowed to proceed for 90 min in a cell culture incubator (at 37 °C under 5% CO_2) in response to different receptor agonists present in the lower compartment separated from the cells by the filter. The number of neutrophils recovered in the lower chamber was determined through the measurement of the MPO activity.[45] The FPR agonists fMLF (10 nM) and WKYMVM (50 nM) were used as positive controls, while buffer was used as a negative control (i.e., spontaneous migration).

Data Analysis. Data analysis was carried out with GraphPad Prism 7.0 (Graphpad Software, San Diego, CA, USA). Curve fitting was performed by nonlinear regression using the sigmoidal dose−response equation (variable-slope). One-way ANOVA and Dunnett's multiple or paired Student's *t*-test were employed for statistical analysis, and the method used is given in the figure legends; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Each independent experiment was performed with neutrophils isolated from different individual blood donors or cell lines obtained after different passages.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00098.

E_{max} values for agonistic peptidomimetics; dual FPR selectivity of peptidomimetic **24**; no off-target effects, shown with peptidomimetic **14**; and characterization of peptidomimetics (PDF)

Molecular formula strings (CSV)

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The manuscript was written via contributions from all authors. All authors have approved the final version of the manuscript.

Funding

Sarah Line Skovbakke was supported by the Carlsberg foundation. The Swedish research group was supported by grants from the Swedish Research Council, the King Gustaf V 80-Year Foundation, the Swedish government under the ALF-agreement, the Clas Groschinsky Memorial Foundation, and the Ingabritt and Arne Lundberg Foundation. The funding agencies had no influence on the study design, data collection and analysis, or writing and submission of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank for the valuable suggestions provided by the members of the Phagocyte Research Group; Birgitte Simonsen for purification of peptidomimetics, and Uraiwan N. Adamsen for mass spectrometric analysis of the compounds.

■ ABBREVIATIONS

*β*NLys, *N*-aminobutyl-*β*-alanine; *β*NPhe, *N*-phenylmethyl-*β*-alanine; *β*Nrpe, *N*-(*R*)-1-phenylethyl-*β*-alanine; *β*Nspe, *N*-(*S*)-1-phenylethyl-*β*-alanine; Aoc, 2-aminooctanoic acid; BSA, bovine serum albumin; CHO, chinese hamster ovary; CL, chemiluminescence; Cy5, cyanine-5; CysH, cyclosporine H; Dab, (*S*)-2,3-diaminobutanoic acid; Dec, decanoyl; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylforamide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; FPR, formyl peptide receptor; Fmoc, fluoren-9-yl-methyloxycarbonyl; Fura-2 AM, Fura-2 acetoxymethyl ester; GPCR, G-protein-coupled receptor; HRP, horseradish peroxidase; IP_3, inositol trisphosphate; KRG, Krebs−Ringer phosphate buffer supplemented with glucose; Lau, lauroyl; MPO, myeloperoxidase; Myr, myristoyl; NADPH, nicotinamide adenine dinucleotide phosphate; Nle, norleucine; NMP, *N*-methyl-2-pyrrolidone; OSu, *N*-oxy-succinimidyl; P2RY2, purinergic receptor P2Y2; Pam, palmitoyl; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; RhB, rhodamine B; RPMI, Roswell Park Memorial Institute; TFA, trifluoroacetic acid; TNF-*α*, tumor necrosis factor *α*; UHPLC, ultrahigh-performance liquid chromatography

■ REFERENCES

(1) Ye, R. D.; Boulay, F.; Wang, J. M.; Dahlgren, C.; Gerard, C.; Parmentier, M.; Serhan, C. N.; Murphy, P. M. International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacol. Rev.* **2009**, *61*, 119−161.

(2) Schepetkin, I. A.; Khlebnikov, A. I.; Giovannoni, M. P.; Kirpotina, L. N.; Cilibrizzi, A.; Quinn, M. T. Development of small molecule non-peptide formyl peptide receptor (FPR) ligands and molecular modeling of their recognition. *Curr. Med. Chem.* **2014**, *21*, 1478−1504.

(3) He, H.-Q.; Ye, R. The formyl peptide receptors: diversity of ligands and mechanism for recognition. *Molecules* **2017**, *22*, 455.

(4) Skovbakke, S. L.; Holdfeldt, A.; Forsman, H.; Bylund, J.; Franzyk, H. The role of formyl peptide receptors for immunomodulatory activities of antimicrobial peptides and peptidomimetics. *Curr. Pharm. Des.* **2018**, *24*, 1100−1120.

(5) Li, L.; Chen, K.; Xiang, Y.; Yoshimura, T.; Su, S.; Zhu, J.; Bian, X.-w.; Wang, J. M. New development in studies of formyl-peptide receptors: critical roles in host defense. *J. Leukoc. Biol.* **2015**, *99*, 425−435.

(6) Dahlgren, C.; Gabl, M.; Holdfeldt, A.; Winther, M.; Forsman, H. Basic characteristics of the neutrophil receptors that recognize formylated peptides, a danger-associated molecular pattern generated by bacteria and mitochondria. *Biochem. Pharmacol.* **2016**, *114*, 22−39.

(7) Jahnsen, R. D.; Haney, E. F.; Franzyk, H.; Hancock, R. E. W. Characterization of a proteolytically stable multifunctional host defense peptidomimetic. *Chem. Biol.* **2013**, *20*, 1286−1295.

(8) Jahnsen, R. D.; Frimodt-Møller, N.; Franzyk, H. Antimicrobial activity of peptidomimetics against multidrug-resistant *Escherichia coli*: a comparative study of different backbones. *J. Med. Chem.* **2012**, *55*, 7253−7261.

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

(9) Olsen, C. A.; Ziegler, H. L.; Nielsen, H. M.; Frimodt-Møller, N.; Jaroszewski, J. W.; Franzyk, H. Antimicrobial, hemolytic, and cytotoxic activities of β-peptoid-peptide hybrid oligomers: improved properties compared to natural AMPs. *ChemBioChem* **2010**, *11*, 1356−1360.
(10) Pupo, A. S.; Duarte, D. A.; Lima, V.; Teixeira, L. B.; Parreiras-e-Silva, L. T.; Costa-Neto, C. M. Recent updates on GPCR biased agonism. *Pharmacol. Res.* **2016**, *112*, 49−57.
(11) Wisler, J. W.; Xiao, K.; Thomsen, A. R.; Lefkowitz, R. J. Recent developments in biased agonism. *Curr. Opin. Cell Biol.* **2014**, *27*, 18−24.
(12) DeFea, K. A. β-Arrestins as regulators of signal termination and transduction: how do they determine what to scaffold? *Cell. Signalling* **2011**, *23*, 621−629.
(13) DeWire, S. M.; Ahn, S.; Lefkowitz, R. J.; Shenoy, S. K. β-Arrestins and cell signaling. *Annu. Rev. Physiol.* **2007**, *69*, 483−510.
(14) Reiter, E.; Ahn, S.; Shukla, A. K.; Lefkowitz, R. J. Molecular mechanism of β-arrestin-biased agonism at seven-transmembrane receptors. *Annu. Rev. Pharmacol. Toxicol.* **2012**, *52*, 179−197.
(15) Whalen, E. J.; Rajagopal, S.; Lefkowitz, R. J. Therapeutic potential of β-arrestin- and G protein-biased agonists. *Trends Mol. Med.* **2011**, *17*, 126−139.
(16) Gabl, M.; Holdfeldt, A.; Sundqvist, M.; Lomei, J.; Dahlgren, C.; Forsman, H. FPR2 signaling without β-arrestin recruitment alters the functional repertoire of neutrophils. *Biochem. Pharmacol.* **2017**, *145*, 114−122.
(17) Holdfeldt, A.; Skovbakke, S. L.; Winther, M.; Gabl, M.; Nielsen, C.; Perez-Gassol, I.; Larsen, C. J.; Wang, J. M.; Karlsson, A.; Dahlgren, C.; Forsman, H.; Franzyk, H. The lipidated peptidomimetic Lau-[(S)-Aoc]-(Lys-β$Nphe)$_6$-$NH_2$ is a novel formyl peptide receptor 2 agonist that activates both human and mouse neutrophil NADPH-oxidase. *J. Biol. Chem.* **2016**, *291*, 19888−19899.
(18) Skovbakke, S. L.; Heegaard, P. M. H.; Larsen, C. J.; Franzyk, H.; Forsman, H.; Dahlgren, C. The proteolytically stable peptidomimetic Pam-(Lys-β$NSpe)$_6$-$NH_2$ selectively inhibits human neutrophil activation via formyl peptide receptor 2. *Biochem. Pharmacol.* **2015**, *93*, 182−195.
(19) Skovbakke, S. L.; Holdfeldt, A.; Nielsen, C.; Hansen, A. M.; Perez-Gassol, I.; Dahlgren, C.; Forsman, H.; Franzyk, H. Combining elements from two antagonists of formyl peptide receptor 2 generates more potent peptidomimetic antagonists. *J. Med. Chem.* **2017**, *60*, 6991−6997.
(20) Christophe, T.; Karlsson, A.; Dugave, C.; Rabiet, M.-J.; Boulay, F.; Dahlgren, C. The synthetic peptide Trp-Lys-Tyr-Met-Val-Met-NH_2 specifically activates neutrophils through FPRL1/lipoxin A4 receptors and is an agonist for the orphan monocyte-expressed chemoattractant receptor FPRL2. *J. Biol. Chem.* **2001**, *276*, 21585−21593.
(21) Freer, R. J.; Day, A. R.; Radding, J. A.; Schiffmann, E.; Aswanikumar, S.; Showell, H. J.; Becker, E. L. Further studies on the structural requirements for synthetic peptide chemoattractants. *Biochemistry* **1980**, *19*, 2404−2410.
(22) Quehenberger, O.; Prossnitz, E. R.; Cavanagh, S. L.; Cochrane, C. G.; Ye, R. D. Multiple domains of the N-formyl peptide receptor are required for high-affinity ligand binding. Construction and analysis of chimeric N-formyl peptide receptors. *J. Biol. Chem.* **1993**, *268*, 18167−18175.
(23) Forsman, H.; Andreasson, E.; Karlsson, J.; Boulay, F.; Rabiet, M.-J.; Dahlgren, C. Structural characterization and inhibitory profile of formyl peptide receptor 2 selective peptides descending from a PIP2-binding domain of gelsolin. *J. Immunol.* **2012**, *189*, 629−637.
(24) Wenzel-Seifert, K.; Seifert, R. Cyclosporin H is a potent and selective formyl peptide receptor antagonist. Comparison with N-t-butoxycarbonyl-L-phenylalanyl-L-leucyl-L-phenylalanyl-L-leucyl-L-phenylalanine and cyclosporins A, B, C, D, and E. *J. Immunol.* **1993**, *150*, 4591−4599.
(25) Forsman, H.; Bylund, J.; Oprea, T. I.; Karlsson, A.; Boulay, F.; Rabiet, M.-J.; Dahlgren, C. The leukocyte chemotactic receptor FPR2, but not the closely related FPR1, is sensitive to cell-penetrating pepducins with amino acid sequences descending from the third intracellular receptor loop. *Biochim. Biophys. Acta* **2013**, *1833*, 1914−1923.
(26) Winther, M.; Gabl, M.; Welin, A.; Dahlgren, C.; Forsman, H. A neutrophil inhibitory pepducin derived from FPR1 expected to target FPR1 signaling hijacks the closely related FPR2 instead. *FEBS Lett.* **2015**, *589*, 1832−1839.
(27) Kenneth, E.; Carlson, T. J. M.; Hunt, S. W. Pepducins: lipopeptide allosteric modulators of GPCR signaling. *Drug Discovery Today: Technol.* **2012**, *9*, e33−e39.
(28) Miller, J.; Agarwal, A.; Devi, L. A.; Fontanini, K.; Hamilton, J. A.; Pin, J.-P.; Shields, D. C.; Spek, C. A.; Sakmar, T. P.; Kuliopulos, A.; Hunt, S. W., 3rd. Insider access: pepducin symposium explores a new approach to GPCR modulation. *Ann. N.Y. Acad. Sci.* **2009**, *1180*, E1−E12.
(29) Winther, M.; Dahlgren, C.; Forsman, H. Formyl peptide receptors in mice and men: similarities and differences in recognition of conventional ligands and modulating lipopeptides. *Basic Clin. Pharmacol. Toxicol.* **2018**, *122*, 191−198.
(30) He, H.-Q.; Troksa, E. L.; Caltabiano, G.; Pardo, L.; Ye, R. D. Structural determinants for the interaction of formyl peptide receptor 2 with peptide ligands. *J. Biol. Chem.* **2014**, *289*, 2295−2306.
(31) Heit, B.; Tavener, S.; Raharjo, E.; Kubes, P. An intracellular signalling hierarchy determines direction of migration in opposing chemotactic gradients. *J. Cell Biol.* **2002**, *159*, 91−102.
(32) Holdfeldt, A.; Dahlstrand Rudin, A.; Gabl, M.; Rajabkhani, Z.; König, G. M.; Kostenis, E.; Dahlgren, C.; Forsman, H. Reactivation of Gαi-coupled formyl peptide receptors is inhibited by Gαq-selective inhibitors when induced by signals generated by the platelet-activating factor receptor. *J. Leukoc. Biol.* **2017**, *102*, 871−880.
(33) Forsman, H.; Önnheim, K.; Andréasson, E.; Christenson, K.; Karlsson, A.; Bylund, J.; Dahlgren, C. Reactivation of desensitized formyl peptide receptors by platelet activating factor: a novel receptor cross talk mechanism regulating neutrophil superoxide anion production. *PLoS One* **2013**, *8*, No. e60169.
(34) Gabl, M.; Holdfeldt, A.; Winther, M.; Oprea, T.; Bylund, J.; Dahlgren, C.; Forsman, H. A pepducin designed to modulate P2Y2R function interacts with FPR2 in human neutrophils and transfers ATP to an NADPH-oxidase-activating ligand through a receptor cross-talk mechanism. *Biochim. Biophys. Acta* **2016**, *1863*, 1228−1237.
(35) Gabl, M.; Winther, M.; Skovbakke, S. L.; Bylund, J.; Dahlgren, C. A pepducin derived from the third intracellular loop of FPR2 is a partial agonist for direct activation of this receptor in neutrophils but a full agonist for cross-talk triggered reactivation of FPR2. *PLoS One* **2014**, *9*, No. e109516.
(36) Önnheim, K.; Christenson, K.; Gabl, M.; Burbiel, J. C.; Müller, C. E.; Oprea, T. I.; Bylund, J.; Dahlgren, C.; Forsman, H. A novel receptor cross-talk between the ATP receptor P2Y2 and formyl peptide receptors reactivates desensitized neutrophils to produce superoxide. *Exp. Cell Res.* **2014**, *323*, 209−217.
(37) Qin, C. X.; May, L. T.; Li, R.; Cao, N.; Rosli, S.; Deo, M.; Alexander, A. E.; Horlock, D.; Bourke, J. E.; Yang, Y. H.; Stewart, A. G.; Kaye, D. M.; Du, X.-J.; Sexton, P. M.; Christopoulos, A.; Gao, X.-M.; Ritchie, R. H. Small-molecule-biased formyl peptide receptor agonist compound 17b protects against myocardial ischaemia-reperfusion injury in mice. *Nat. Commun.* **2017**, *8*, 14232.
(38) Bylund, J.; Björstad, Å.; Granfeldt, D.; Karlsson, A.; Woschnagg, C.; Dahlgren, C. Reactivation of formyl peptide receptors triggers the neutrophil NADPH-oxidase but not a transient rise in intracellular calcium. *J. Biol. Chem.* **2003**, *278*, 30578−30586.
(39) Jesaitis, A. J.; Klotz, K. N. Cytoskeletal regulation of chemotactic receptors: molecular complexation of N-formyl peptide receptors with G proteins and actin. *Eur. J. Haematol.* **1993**, *51*, 288−293.
(40) Seifert, R. Functional selectivity of G-protein-coupled receptors: from recombinant systems to native human cells. *Biochem. Pharmacol.* **2013**, *86*, 853−861.

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982

(41) Bonke, G.; Vedel, L.; Witt, M.; Jaroszewski, J. W.; Olsen, C. A.; Franzyk, H. Dimeric building blocks for solid-phase synthesis of α-peptide-β-peptoid chimeras. *Synthesis-Stuttgart* **2008**, 2381−2390.
(42) Boyum, A.; Lovhaug, D.; Tresland, L.; Nordlie, E. M. Separation of leucocytes: improved cell purity by fine adjustments of gradient medium density and osmolality. *Scand. J. Immunol.* **1991**, *34*, 697−712.
(43) Dahlgren, C.; Karlsson, A. Respiratory burst in human neutrophils. *J. Immunol. Methods* **1999**, *232*, 3−14.
(44) Bylund, J.; Björnsdottir, H.; Sundqvist, M.; Karlsson, A.; Dahlgren, C. Measurement of respiratory burst products, released or retained, during activation of professional phagocytes. *Methods Mol. Biol.* **2014**, *1124*, 321−338.
(45) Somersalo, K.; Salo, O. P.; Björkstén, F.; Mustakallio, K. K. A simplified Boyden chamber assay for neutrophil chemotaxis based on quantification of myeloperoxidase. *Anal. Biochem.* **1990**, *185*, 238−242.

DOI: 10.1021/acsomega.9b00098
ACS Omega 2019, 4, 5968−5982